Chemical and enzymatic stability of amino acid derived phosphoramidates of antiviral nucleoside 5′-monophosphates bearing a biodegradable protecting group†

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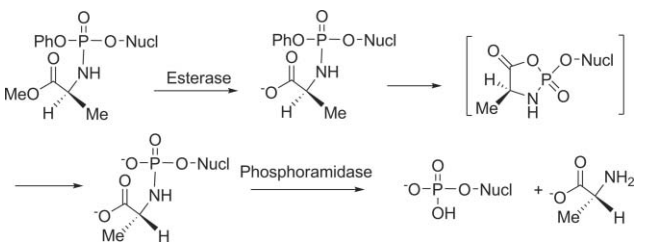
Ribavirin and 2′-*O*-methylcytidine 5′-phosphoramidates derived from L-alanine methyl ester bearing either an *O*-phenyl or a biodegradable *O*-[3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl] or *O*-[3-(acetyloxymethoxy)-2,2-bis(ethoxycarbonyl)propyl] protecting group were prepared. The kinetics of the deprotection of these pro-drugs by porcine liver esterase and by a whole cell extract of human prostate carcinoma was studied by HPLC-ESI-MS/MS. The 3-(acetyloxymethoxy)-2,2-bis(ethoxycarbonyl)propyl and 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl groups were readily removed releasing the L-alanine methyl ester phosphoramidate nucleotide, the deprotection of the 3-(acetyloxymethoxy) derivative being approximately 20 times faster. The chemical stability of the 2′-*O*-methylcytidine pro-drugs was additionally determined over a pH range from 7.5 to 10.

Introduction

Many of the currently used antiviral drugs are uncharged structural analogs of nucleosides.[1] Their biological activity, however, often depends on conversion into 5′-mono-, 5′-di- or 5′-triphosphates by intracellular kinases,[2] the first phosphorylation being in several cases the rate-limiting step in human cells.[3] Direct administration of the corresponding nucleotides should bypass this limiting step and lead to improved biological activity. Owing to their ionic character, nucleoside monophosphates are, however, unable to penetrate cellular membranes, and hence, masking of the phosphate moiety with biodegradable lipophilic protecting groups has received increasing interest during the past decade.[4]

The key feature of most nucleotide pro-drugs is that the negatively charged phosphate group remains masked outside the cell and becomes exposed by intracellular enzymes. A pro-drug strategy may, however, serve two additional purposes: (i) a biodegradable protecting group bearing an appropriate conjugate group may allow receptor-mediated endocytosis of the pro-drug and (ii) chemically controlled departure of the protecting group after the initial enzymatic activation allows tuning of the release rate of the active drug. Modified nucleoside 5′-(*O*-arylphosphoramidate)s derived from the methyl esters of naturally occurring amino acids constitute an extensively studied class of pronucleotides,[5] which exhibit two of the important characteristics of pro-drugs. The lipophilic aryl group masks the negative charge, while the amino acid ester moiety allows receptor mediated endocytosis. In fact, the amino acid ester moiety has been shown to be advantageous for the biological activity, L-alanine and L-tryptophan exhibiting the most pronounced antiviral activity.[6] Inside the cell, esterase catalyzed hydrolysis of the carboxylic ester linkage is believed to result in cleavage of the aryl group by an intramolecular attack of the carboxylate group on the phosphorus atom (Scheme 1).[7] Hydrolysis of the cyclic structure then gives the *N*-substituted nucleoside 5′-phosphoramidate monoanion,[7,8] and phosphoramidase is believed to release the 5′-monophosphate.[7,9]



Scheme 1 Biodegradation of nucleoside 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate}s.

This concept has been applied to a wide variety of nucleotides and their phosphonate congeners: AZTMP[10] and its analogs,[11] d4TMP,[6a,12] dioxolane-TMP,[13] ddAMP,[14] d4AMP,[14,15] carbocyclic L-d4AMP,[16] abacavir phosphate,[17] ddUMP,[18] 4′-azido-NMPs,[19] 6-hydrazinopurine 2′-methyl ribonucleoside 5′-phosphate[20] and acyclic nucleoside phosphonates[21] and phosphates.[22] Interestingly, amino acid phosphoramidate nucleotides can themselves serve as alternative substrates for HIV-1 reverse transcriptase.[23] Accordingly, the antiviral effect does not always necessarily depend on conversion of the 5′-phosphoramidic acid to 5′-phosphate, but the 5′-phosphoramidic acid itself may be incorporated into the DNA of the host by a reverse transcriptase. The pro-drug strategies of

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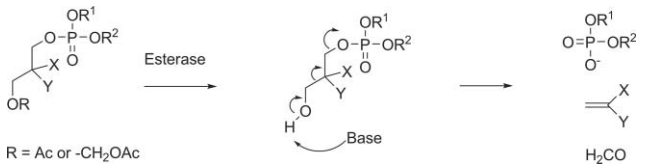
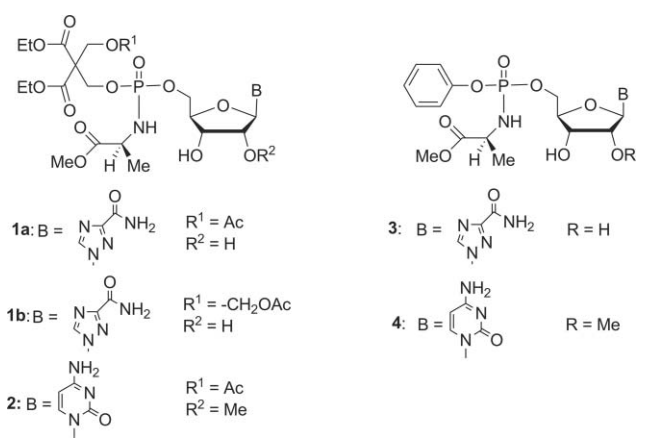
nucleoside 5′-phosphoramidic acids, hence, are of considerable interest.

We have previously shown that appropriate 2,2-disubstituted 3-acyloxypropyl[24] and 3-acyloxymethoxypropyl[25] groups are viable biodegradable protecting groups for phosphodiesters. A useful feature of this kind of protecting group is that the stability of the product obtained by the enzymatic deprotection may be varied within wide limits by the polar nature of the 2-substituents and conjugate groups may be attached *via* ester and amide linkages to C2 of the 3-acyloxypropyl group without interfering with the departure of the group by esterase-triggered concerted retro-aldol condensation and phosphate elimination (Scheme 2).[26] On the other hand, the identity of the acyl group has a marked effect on the rate of the enzymatic deprotection step. We now report on our studies aimed at applying this kind of group, 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl and 3-(acetyloxymethoxy)-2,2-bis(ethoxycarbonyl)propyl groups, to the biodegradable protection of nucleoside 5′-phosphoramidates.



Scheme 2 Esterase triggered removal of 2,2-disubstituted 3-acetyloxy- and 3-acetyloxymethoxypropyl protected phosphoesters.

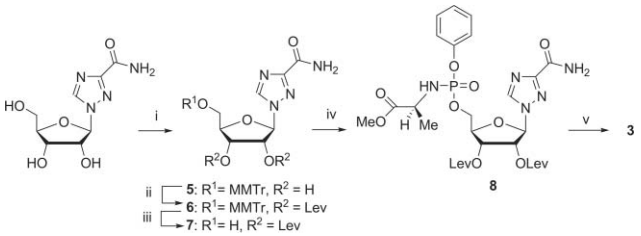
For this purpose, two antiviral nucleoside analogs, *viz*. ribavirin and 2′-*O*-methylcytidine, were converted to 5′-phosphoramidates derived from L-alanine methyl ester and bearing either an *O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl] (**1a**, **2**), *O*-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl] (**1b**) or *O*-phenyl (**3**, **4**) group. The release of the respective 5′-phosphoramidic acids upon treatment with porcine liver carboxyesterase (PLE) or a whole cell extract of human prostate carcinoma cells (PC3) was studied. The latter studies were undertaken to find out whether the 2,2-bis(ethoxycarbonyl)propyl-derived groups afford viable pro-drug candidates of phosphoramidates that might be worth detailed biological studies and could possibly offer an alternative for the extensively used *O*-phenyl phosphoramidates.



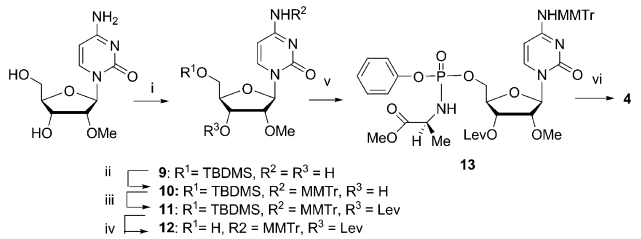
Results and discussion

Syntheses

The 5′-[*O*-phenylphosphoramidates] of ribavirin (**3**) and 2′-*O*-methylcytidine (**4**) were obtained as a mixture of the R_P- and S_P-diastereomers by reaction of the appropriately protected nucleosides with diphenylphosphite in pyridine under nitrogen and subsequent oxidative amination of the 5′-(*H*-phosphonate) phenyl esters with L-alanine methyl ester (Scheme 3 and 4). The levulinoyl protections were removed with hydrazine hydrate treatment in a mixture of acetic acid and pyridine and the N^4-MMTr group with aq AcOH.



Scheme 3 Preparation of ribavirin 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate}: i) MMTrCl, Py, ii) levulinic anhydride, DMAP, Py, iii) 80% AcOH (aq.), iv) diphenylphosphite, L-alanine methyl ester, Py/MeCN, CCl_4, TEA, v) 0.5 mol L^{-1} $N_2H_2{\cdot}H_2O$.

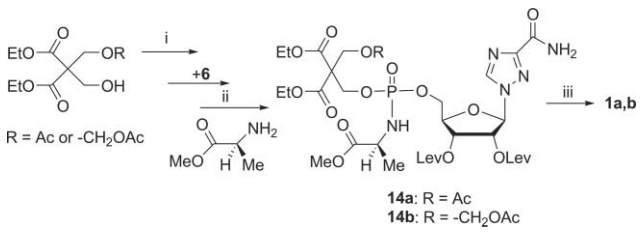


Scheme 4 Preparation of 2′-*O*-methylcytidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate}: i) TBDMSCl, Py, ii) MMTrCl, Py, iii) levulinic anhydride, DMAP, Py, iv) TBAF, THF/AcOH, v) diphenylphosphite, L-alanine methyl ester, Py/MeCN, CCl_4, TEA, vi) 1. 0.5 mol L^{-1} $N_2H_4{\cdot}H_2O$ AcOH/Py, 2. 80% AcOH (aq.).

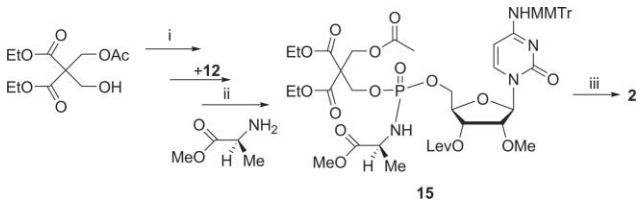
Schemes 5 and 6 outline the synthesis of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl (**1a**, **2**) and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl (**1b**) protected phosphoramidates. Diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate[25] in the case of **1a** and diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate[25] in the case of **1b** was first reacted with diphenylphosphite to obtain a mixed alkyl phenyl H-phosphonate diester, from which phenol was displaced with the appropriately protected nucleoside, either **7** or **12**, according to the method of Zhu *et al.*[27] After 2 h, oxidative amination with L-alanine methyl ester was carried out and the protecting groups were removed.

Hydrolytic stability of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl substituted phosphoramidates

The hydrolytic stability of the *O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl] substituted phosphoramidates was evaluated using the 2′-*O*-methylcytidine derivative, **2**, as a model compound.



Scheme 5 Preparation of ribavirin 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate} (**1a**) and 5′-{*O*-[3-acetyloxymethoxy-2,2-bis-(ethoxycarbonyl)propyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methyl-]phosphoramidate} (**1b**): i) diphenylphosphite, Py, ii) Py/MeCN, CCl_4, TEA, iii) 0.5 mol L^{-1} $N_2H_4{\cdot}H_2O$ AcOH/Py.



Scheme 6 Preparation of 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate}: i) diphenylphosphite, Py, ii) Py/MeCN, CCl_4, TEA, iii) 1. 0.5 mol L^{-1} $N_2H_4{\cdot}H_2O$ AcOH/Py, 2. 80% AcOH (aq.).

The reactions were followed by determining the product composition of the aliquots withdrawn from the reaction solution at appropriate time intervals by RP HPLC. The products were characterized by mass spectrometric analysis (HPLC/ESI-MS). The time-dependent product distribution obtained at pH 10.0 and 37 °C is given in Fig. 1. As seen, two products, *viz.* 2′-*O*-methylcytidine 5′-{*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate} (**17**) and 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate} (**18**), are first formed in parallel. Both of these intermediates are subsequently converted to 2′-*O*-methylcytidine 5′-{*N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate} (**20**). Most likely the pathways indicated in Scheme 7 are followed. The acetyl group of the *O*-linked phosphate protecting group is first hydrolyzed giving **16**. This product is not, however, accumulated, but a rapid retro-aldol condensation followed by elimination to **17** takes place. Alternatively, the methyl ester linkage of the alaninyl moiety is first hydrolyzed yielding **18**. The final product, **20**, is then obtained by the carboxy ester hydrolysis of **17**, or by deacetylation of **18** to **19**, followed by retro-aldol condensation and concomitant phosphate elimination to **20**. The deacetylated intermediate **19** is not accumulated under the experimental conditions. Application of the rate-law for parallel consecutive first-order reactions gives the rate constants k_1, k_3, k_4 and k_5 indicated in Scheme 7. When the acetyl group is removed from **2** by a PLE-treatment prior to the kinetic run, the kinetics of the conversion of **16** to **17** may be studied and the rate constant k_2 is obtained. Evidently k_6 is approximately equal to k_2; replacement of the methoxycarbonyl group in **17** with a carboxy group in **19** hardly has a major effect on the rate of the elimination step. Table 1 records the rate constants obtained.

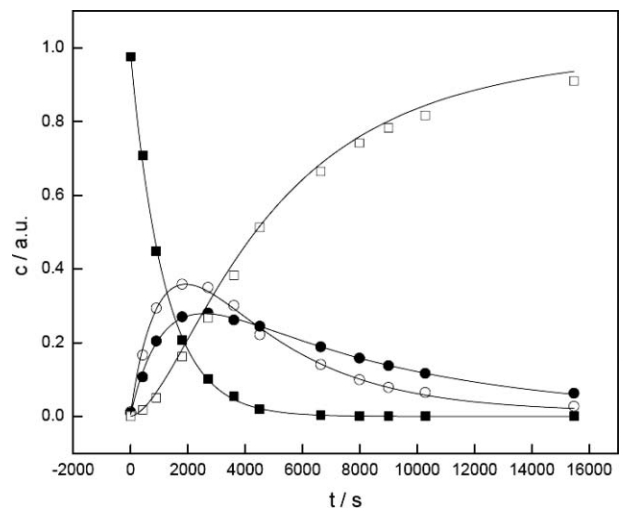
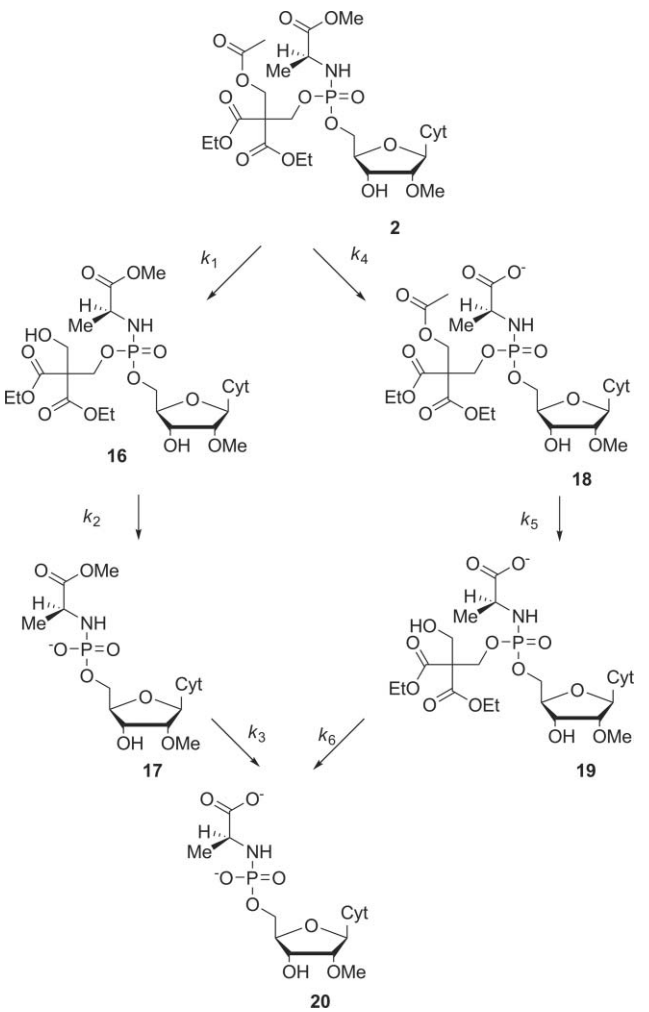


Fig. 1 Time-dependent product distribution for the hydrolysis of 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} 2 at pH 10.0 (0.06 mol L^{-1} glycine buffer) and 37 °C. Notation: (■) **2**, (●) **17**, (○) **18**, (□) **20**. For the structures, see Scheme 7.



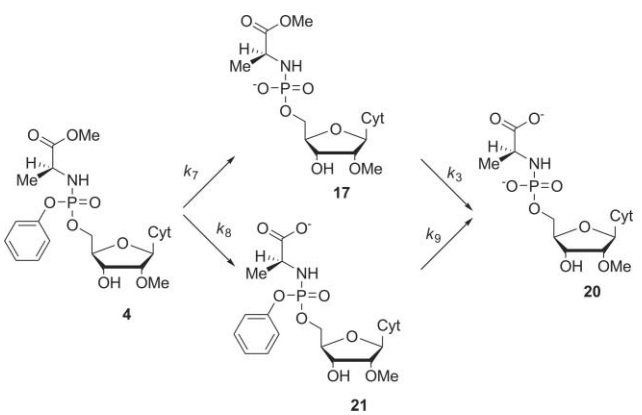
Scheme 7 The non-enzymatic hydrolysis of 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**2**).

Table 1 First-order rate constants for the partial reactions involved in the hydrolysis of compound **2** to **20** at 37.0 °C. For the definition of the rate constants, see Scheme 7

	pH		
	7.5	9.0	10.0
$k_d/10^{-5}s^{-1}$ [a]	0.32 ±0.02	8.9 ± 0.3	84 ± 2
$k_1/10^{-5}s^{-1}$		3.5 ± 0.1	33.0 ± 0.6
$k_2/10^{-5}s^{-1}$	67 ± 1		
$k_3/10^{-5}s^{-1}$		2.0 ± 0.1	13.9 ± 0.4
$k_4/10^{-5}s^{-1}$		5.4 ± 0.2	51 ± 1
$k_5/10^{-5}s^{-1}$		3.4 ± 0.4	29 ± 1

[a] $k_d = k_1 + k_4$.

The kinetics and mechanisms of the non-enzymatic hydrolysis of the thymidine analog of *O*-phenyl pro-drugs **3** and **4** have previously been studied over a wide pH-range at 90 °C.[8] To allow comparison with the hydrolytic stability of **2**, the first-order rate constant for the hydrolysis of **4** was now determined at pH 7.5 and 37 °C. Again two parallel reactions may in principle occur (Scheme 8). Either, hydroxide ion first displaces the phenoxy ligand giving **17**, and the methyl ester linkage is then hydrolyzed. The final product (**20**), hence, is the same as in the hydrolysis of **2**. This product is also obtained by initial cleavage of the methyl ester function of **4**, followed by displacement of phenoxide ion, in all likelihood assisted by the deprotected alanine carboxylate group (*cf*. Scheme 1). The time-dependent composition of the reaction mixture at pH 7.5 is presented in Fig. 2. In contrast to the hydrolysis of **2**, neither of the intermediates **17** and **21** is appreciably accumulated. Our previous studies[8] on the cleavage of the corresponding thymidine derivative suggest that the pathway through **21** is one order of magnitude faster that the reaction through **17**. The assumption that this is the case also with **4** receives considerable support from the fact that the first-order rate constant k_3 may be estimated to be smaller than 10^{-6} s^{-1} at pH 7.5 (37 °C). As indicated in Table 1, the rate constant at a 30-fold higher hydroxide ion concentration is 2×10^{-5} s^{-1}. Since the rate constant for the disappearance of **4** and, hence, for the formation of **20** is $(3.1 \pm 0.1)\times10^{-6}$ s^{-1}, **17** cannot lie on the main reaction pathway, its decomposition to **20** is too slow.



Scheme 8 The non-enzymatic hydrolysis of 2′-*O*-methylcytidine 5′-{*O*-phenyl-*N*-[(*S*)- 2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**4**).

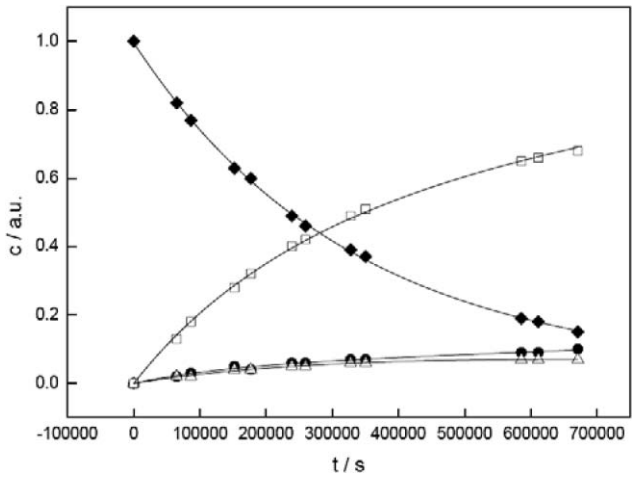


Fig. 2 Time-dependent product distribution for the hydrolysis of 2′-*O*-methylcytidine 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**4**) at pH 7.5 (0.06 M HEPES buffer) and 37.0 °C: (◆) **4**, (●) **17**, (△) **21**, (□) **20**.

Enzymatic hydrolyses of 1-4

Enzymatic removal of protecting groups from the 2′-*O*-methylcytidine pro-drugs **2** and **4** was studied with porcine liver esterase (PLE; 26 unit mL^{-1}) in HEPES buffer (20 mmol L^{-1}, pH 7.5, $I = 0.1$ mol L^{-1} with NaCl) at 35 °C. Fig. 3 and 4 show examples of the HPLC traces obtained for the reaction mixtures at different intervals. The *O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate **2** undergoes two parallel reactions (Fig. 3). The predominant reaction is the conversion of the two diastereomers, R_P-**2** and S_P-**2** (*m*/*z* 666), to *N*-(2-methoxy-1-methyl-2-oxoethyl)phosphoramidate **17** (*m*/*z* 422) *via* intermediary accumulation of the R_P and S_P diastereomers of *O*-[3-hydroxy-2,2-bis(ethoxycarbonyl)-propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate **16** (*m*/*z* 624). One of the two diastereomers seems to be consumed slightly faster than the other, but the data available does not allow us to distinguish which isomer, R_P-**2** or S_P-**2**, reacts more readily. As a side-reaction representing 20% of the overall disappearance of **2**, the alanine ester linkage is hydrolyzed, giving the *O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]-*N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate **18** (*m*/*z* 652). In addition, *N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate **20** (*m*/*z* 408) is formed. This compound may in principle be produced both by the carboxy ester hydrolysis of **17** and by enzymatic deacetylation of **18** followed by retro-aldol condensation and concomitant phosphate elimination. The half-life for the disappearance of **2** is 6.6 h [$k = (3.4 \pm 0.2)\times10^{-5}$ s^{-1}].

With the *O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate (**4**), the only reaction detected is conversion of the diastereomeric mixture of R_P-**4** and S_P-**4** (*m*/*z* 498) to the *N*-(2-hydroxy-1-methyl-2-oxoethyl)phosphoramidate **20** (*m*/*z* 408; Fig. 4). The diastereomers again react at slightly different rates, but which one of them reacts faster, cannot be decided on the basis of the data available. The half-life for the reaction is 43 h [$k = (4.5 \pm 0.4)\times10^{-6}$ s^{-1}]. Accordingly, the reaction of **4** to **20** is almost an order of magnitude slower than the conversion of **2** to **17**. Neither **20** nor **17** exhibited any tendency to be further hydrolyzed to

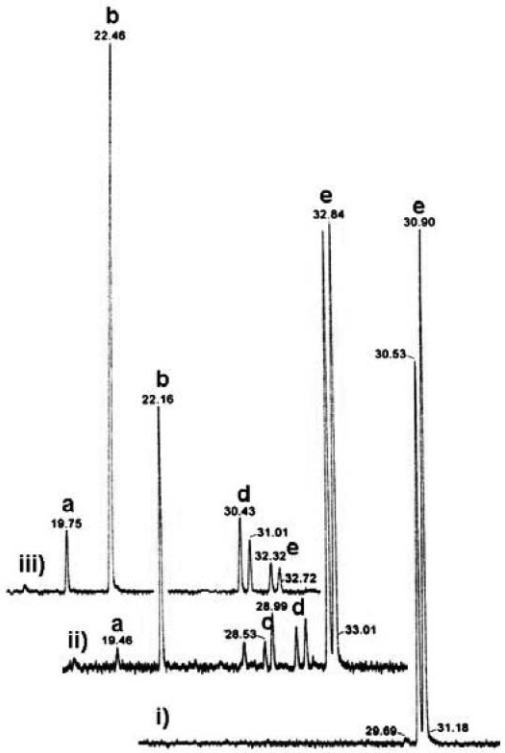


Fig. 3 HPLC traces for the PLE-catalyzed (26 U mL^{-1}) deprotection of 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate (**2**) in HEPES buffer (20 mmol L^{-1}, pH 7.5, $I = 0.1$ mol L^{-1} with NaCl) at 35 °C. Notation: i) at $t = 0$, ii) at $t = 2.5$ h, and iii) at $t = 24$ h; a refers to compd. **20**, b to **17**, c to **16**, d to **18**, and e to **2**. The signal at 28.53 min did not exhibit any detectable $m/z > 150$.

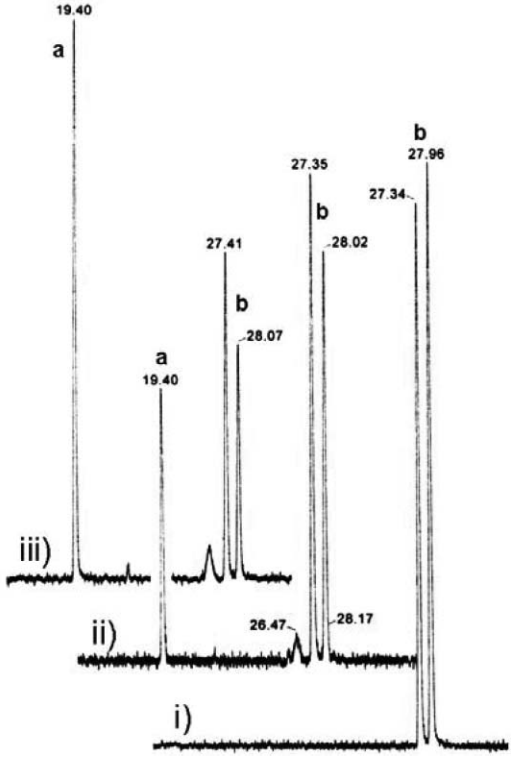
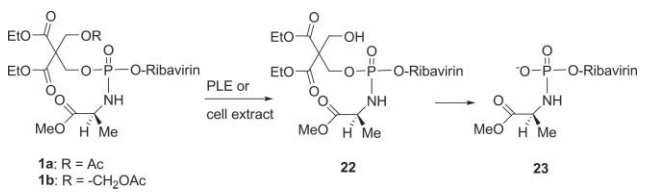


Fig. 4 HPLC traces for the PLE-catalyzed (26 U mL^{-1}) deprotection of 2′-*O*-methylcytidine 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**4**) in HEPES buffer (20 mmol L^{-1}, pH 7.5, $I = 0.1$ mol L^{-1} with NaCl) at 35 °C. Notation: i) at $t = 0$, ii) at $t = 2.5$ h, and iii) at $t = 24$ h; a refers to compd. **20**, and b to **4**. The signal at 26.47 min did not exhibit any detectable $m/z > 150$.

nucleoside 5′-monophosphate. If this were to happen, the process must be enzymatic rather than chemical.

All the protected phosphoramidates, **1-4**, were additionally treated with PLE at a lower enzyme concentration and **1a** and **2-4** with a PC3-cell extract to study the removal of the protecting groups. When the *O*-phenyl protected phosphoramidates, **3** and **4**, were treated with PLE (1 unit mL^{-1}) or PC3-cell extract at pH 7.5 and 37 °C, the disappearance of the starting materials was not appreciably accelerated compared to the non-enzymatic hydrolysis. The same experiments were then carried out with **1a** and **2**. After one day, 36% of **1a** was decomposed when incubated with PLE and 48% in PC3 cell extract. The product distribution was analyzed by HPLC and the isolated peaks were identified by ESI-TOF-MS analysis. Both in the presence of PLE and in PC3-cell extract, the main product of **1a** was phosphoramidate **23** ($[M - H]^-$ m/z 408; Scheme 9). When **1b** was incubated at low PLE concentration (1 unit mL^{-1}), more than 78% of **1b** was decomposed in six hours. The product distribution was analyzed by HPLC and the isolated peaks were subjected to ESI-TOF-MS analysis. The main product of **1b** was phosphoramidate **23** ($[M - H]^-$ m/z 408; Scheme 9). The enzymatic deacetylation of the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group from **1b** is, hence, 20-fold faster than the deacetylation of its 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-protected counterpart



Scheme 9 Hydrolysis of ribavirin 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**1a**) and 5′-{*O*-[3-acetyloxy-methoxy-2,2-bis(ethoxycarbonyl)-propyl-*N*-[(*S*)-2-oxo-2-methoxy-1-methyl]phosphoramidate} (**1b**) in the presence of PLE or in cell extract.

1a. The situation is similar to that reported previously for deacetylation of the corresponding 5′-phosphotriesters.[25]

Compound **2**, the 2′-*O*-methylcytidine analog of **1a**, underwent a somewhat slower deprotection to **20** in the PC3-cell extract (Fig. 5). Owing to the slower rate of initial deacetylation compared to the PLE-catalyzed reaction depicted in Fig. 3, compound **16** did not accumulate, but underwent a subsequent retro-aldol condensation and concomitant phosphate elimination to **17**. In addition, the starting material underwent hydrolysis of the alaninyl ester giving the two diastereomers of **18**. Both **17** and **18** eventually gave **20**, **18** faster than **17**.

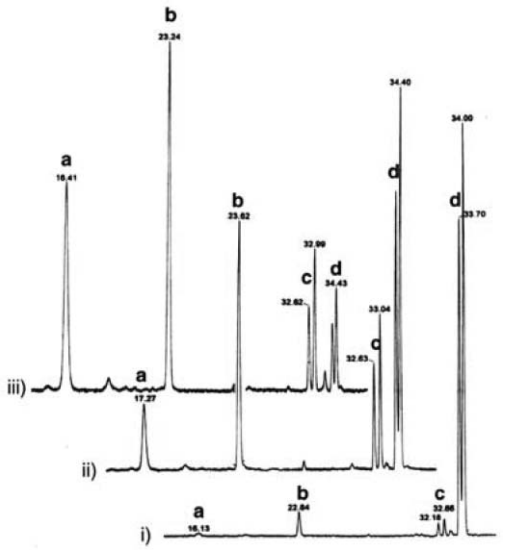


Fig. 5 HPLC traces for deprotection of 2′-*O*-methylcytidine 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate (**2**) in PC3-cell extract diluted with a double volume of HEPES buffer (20 mmol L^{-1}, pH 7.5, $I = 0.1$ mol L^{-1} with NaCl, $T = 35$ °C). Notation: i) at $t = 1$ d, ii) at $t = 6$ d, and iii) at $t = 18$ d; a refers to compd. **20**, b to **17**, c to **18**, and d to **2**.

Experimental section

Syntheses

5′-*O*-(4-Methoxytrityl)ribavirin (5). Ribavirin (8.31 mmol; 2.03 g) was dried by repeated coevaporations from dry pyridine and dissolved in the same solvent (15 mL). 4-Methoxytrityl chloride (8.32 mmol; 2.57 g) was added and the reaction was allowed to proceed overnight. The mixture was evaporated to dryness and the residue was equilibrated between chloroform and water. The organic phase was dried on Na_2SO_4. The crude product was purified by silica gel chromatography using gradient elution from 5 to 10% MeOH in DCM. Yield 68%. ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H, H5), 7.39-741 (m, 4H, MMTr), 7.27-7.30 (m, 2H, MMTr), 7.21-7.24 (m, 4H, MMTr), 7.15-7.18 (m, 2H, MMTr), 7.09 (br s, 1H, NH), 6.78-6.80 (m, 2H, MMTr), 6.43 (br s, 1H, NH), 5.98 (d, $J = 3.5$ Hz, 1H, H1′), 4.79 (dd, $J = 3.5$ and 4.7 Hz, 1H, H2′), 4.48 (dd, $J = 4.7$ and 5.1, 1H, H3′), 4.31 (m, 1H, H4′), 3.73 (s, 3H, MeO-MMTr), 3.43 (dd, $J = 10.6$ and 2.8 Hz; 1H, H5′), 3.31 (dd, 10.6 and 4.3 Hz, 1H, H5″). ^{13}C NMR ($CDCl_3$) δ 161.3 (C=O), 158.6 (MMTr), 156.5 (C3), 144.6 (MMTr), 144.0 (C5), 136.3 (MMTr), 130.4 (MMTr), 128.3 (MMTr), 127.9 (MMTr), 127.0 (MMTr), 113.2 (MMTr), 92.9 (C1′), 86.7 (MMTr), 84.6 (C4′), 75.3 (C2′), 71.1 (C3′), 63.5 (C5′), 55.2 (MMTr). ESI-HRMS: $[M + Na]^+$ obsd. 539.1852, calcd. 539.1909.

2′,3′-Di-*O*-levulinoyl-5′-*O*-(4-methoxytrityl)ribavirin (6). Levulinic acid (28.3 mmol; 3.29 g) was dissolved in dry dioxane and the solution was cooled to 0 °C on an ice bath. Dicyclohexylcarbodiimide (14.2 mmol; 2.93 g) was added portion wise during 1 h. Dicyclohexylurea formed was removed by filtration. The filtrate and the dioxane washing of the precipitate (5 mL) were combined and mixed with the solution of compd. **5** (dried on P_2O_5) in dry pyridine (15 mL). A catalytic amount of 4-dimethylaminopyridine was added and the reaction was allowed to proceed overnight. Volatiles were removed under reduced pressure and the residue was subjected to DCM/aq $NaHCO_3$ work-up. The organic phase was dried on Na_2SO_4. The crude product (**6**) was used as such for the removal of the monomethoxytrityl protection. ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1H, H5), 7.42-7.44 (m, 4H, MMTr), 7.23-7.32 (m, 8H, MMTr), 6.78-6.80 (m, 2H, MMTr), 6.66 (br s, 1H, NH), 6.08 (d, $J = 4.9$ Hz, 1H, H1′), 6.00 (dd, $J = 4.9$ and 5.3 Hz, 1H, H2′), 5.69 (br s, 1H, NH), 5.63 (dd, $J = 4.1$ and 5.3, 1H, H3′), 4.40 (m, 1H, H4′), 3.80 (s, 3H, MeO-MMTr), 3.47 (dd, $J = 10.7$ and 2.8 Hz; 1H, H5′), 3.36 (dd, 10.7 and 4.3 Hz, 1H, H5″), 2.76-2.81 (m, 4H, Lev), 2.61-2.67 (m, 4H, Lev), 2.20 (s, 6H, Lev). ^{13}C NMR ($CDCl_3$) δ 206.3 (2×C=O Lev), 171.6 (C=O Lev), 171.3 (C=O Lev), 160.3 (C=O), 158.7 (MMTr), 157.2 (C3), 144.7 (MMTr), 143.7 (C5), 136.0 (MMTr), 130.5 (MMTr), 128.4 (MMTr), 128.0 (MMTr), 127.2 (MMTr), 113.2 (MMTr), 89.9 (C1′), 87.2 (MMTr), 83.1 (C4′), 74.3 (C2′), 71.4 (C3′), 62.9 (C5′), 55.2 (MMTr), 37.8 (Lev), 37.7 (Lev), 29.8 (Lev), 29.7 (Lev), 27.6 (Lev), 27.5 (Lev). ESI-HRMS: $[M + Na]^+$ obsd. 735.2595, calcd. 735.2644.

2′,3′-Di-*O*-levulinoylribavirin (7). Compd. **6** (7.17 mmol; 5.11 g) was treated with 80% aq AcOH (100 mL) overnight. The mixture was evaporated to dryness and the residue was purified by silica gel chromatography using gradient elution from 5 to 10% MeOH in DCM. Yield from **5** 66%. ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 8.60 (s, 1H, H5), 7.34 (s, 1H, NH), 6.08 (d, $J = 4.3$ Hz, 1H, H1′), 5.71 (dd, $J = 4.3$ and 5.2 Hz, 1H, H2′), 5.58 (dd, $J = 4.3$ and 5.2, 1H, H3′), 5.32 (s, 1H, NH), 4.35 (m, 1H H4′), 3.91 (dd, $J = 12.7$ and 2.4 Hz, 1H, H5′), 3.77 (dd, $J = 12.7$ and 2.8 Hz, 1H, H5″), 2.77-2.82 (m, 4H, Lev), 2.60-2.67 (m, 4H, Lev), 2.21 (s, 3H, Lev), 2.19 (s, 3H, Lev). ^{13}C NMR ($CDCl_3$ + CD_3OD) δ 207.0 (C=O Lev), 171.9 (C=O Lev), 171.4 (C=O Lev), 161.0 (C=O), 157.0 (C3), 144.7 (C5), 90.4 (C1′), 84.7 (C4′), 75.1 (C2′), 71.1 (C3′), 61.0 (C5′), 37.7 (Lev), 37.6 (Lev), 29.8 (Lev), 29.7 (Lev), 27.5 (Lev), 27.4 (Lev). ESI-HRMS: $[M + H]^+$ obsd. 441.1581, calcd. 441.1623; $[M + Na]^+$ obsd. 463.1401, calcd. 463.1443.

2′,3′-Di-*O*-levulinoylribavirin 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate} (8). Comp. **7** (0.41 mmol; 0.18 g) was coevaporated twice from dry pyridine, dissolved in the same solvent (3.0 mL) and diphenylphosphite (0.61 mmol; 118 μL) was added under nitrogen. After 20 min, L-alanine methyl ester (0.86 mmol; 0.12 g) dried by coevaporation from pyridine was added dissolved in a mixture of dry MeCN (4.0 mL) and pyridine (1.0 mL). Immediately after this addition, CCl_4 (2.5 mL) and distilled triethylamine (2.8 mmol; 400 μL) were added. The reaction was allowed to proceed for 70 min and the mixture was then evaporated to dryness. Silica gel chromatography by a gradient elution from 3 to 10% of MeOH in DCM gave compd. **8** as with foam in 67% yield. ^{1}H NMR ($CDCl_3$) mixture of R_P and S_P diastereomers δ 8.40 and 8.46 (2×s, 1H, H5), 7.34 and 7.37 (2×br s, 1H, NH_2), 7.10-7.30 (m, 5H, Ph), 6.38 and 6.46 (2×br s, 1H, NH_2), 6.09 and 6.10 (2×d, $J = 4.5$ Hz, 1H, H1′), 5.71 and 5.73 (2×dd, $J = 4.5$ and 5.0, 1H, H2′), 5.61 and 5.63 (2×dd, $J = 5.0$ and 5.0, 1H, H3′), 4.45-4.49 (m, 1H, H4′), 4.28-4.43 (m, 2H, H5′ and H5″), 3.98-4.07 (m, 1H, H^{α}-Ala), 3.62 and 3.64 (2×s, 3H, MeO-Ala), 2.73-2.79 (m, 4H, Lev), 2.56-2.66 (m, 4H, Lev), 2.17 and 2.18 (2×s, 6H, Lev), 1.31 and 1.33 (2×d, $J = 7.2$ Hz, 3H, Me Ala). ^{31}P NMR ($CDCl_3$) δ 3.0 and 3.2. ESI-HRMS: $[M + H]^+$ obsd. 682.2063, calcd. 682.2127; $[M + Na]^+$ obsd. 704.1918, calcd. 704.1947.

Ribavirin 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (3). Compd. **8** (0.21 mmol; 0.14 g) was dissolved at 0 °C into a mixture of hydrazine hydrate (4.0 mmol; 124 μL), dry pyridine (4.0 mL) and AcOH (1.0 mL) and the reaction was allowed to proceed for 1 h. The unreacted hydrazine was quenched with acetone. The volatiles were removed under reduced pressure and the crude product was purified by silica gel chromatography, increasing the MeOH content of DCM in a stepwise manner from 5% to 10% and then to 20%. Yield 60%. ^{1}H NMR (CD_3OD) mixture of R_P and S_P diastereomers δ 8.72 and 8.74 (2×s, 1H, H5), 7.33-7.37 (m, 2H, Ph), 7.17-7.23 (m, 3H, Ph), 5.98 (2×d, J = 3.5 Hz, 1H, H1′), 4.54 and 4.56 (2×dd, J = 3.5 and 4.7 Hz, 1H, H2′), 4.47 (dd, J = 4.7 and 5.9, 1H, H3′), 4.26-4.43 (m, 3H, H4′, H5′ and H5″), 3.91 and 3.94 (2×dd, J = 9.3 and 7.2 Hz, H$^{\alpha}$-Ala), 3.65 and 3.67 (2×s, 3H, MeO-Ala), 1.29 and 1.32 (2×d, J = 7.2 Hz, 3H, Me Ala). ^{13}C NMR (CD_3OD) δ 174.1 (C=O Ala), 161.9 ($CONH_2$), 157.1 (Ph), 150.7 (C3), 145.3 (C5), 92.4 (C1′), 83.1 (C4′), 74.8 (C2′), 70.2 (C3′), 65.9 (C5′), 51.9 (MeO-Ala), 49.8 (C$^{\alpha}$-Ala), 19.1 (Me Ala). ^{31}P NMR (CD_3OD) δ 3.8 and 4.0. ESI-HRMS: $[M + H]^+$ obsd. 486.1389, calcd. 486.1384; $[M + Na]^+$ obsd. 508.1206, calcd. 508.1204; $[M + K]^+$ obsd. 524.0937, calcd. 524.0943.

5′-*O*-(*tert*-Butyldimethylsilyl)-2′-*O*-methylcytidine (9). 2′-*O*-Methylcytidine (18.4 mmol; 4.74 g; PI Chemicals) was coevaporated twice from dry pyridine, dried over P_2O_5 (24 h) and dissolved in dry pyridine (20 mL). *tert*-Butyldimethylsilyl chloride (TBDMSCl; 20.2 mmol; 3.05 g) was added and the mixture was agitated at room temperature overnight. The unreacted TBDMSCl was quenched with MeOH, the mixture was evaporated to dryness and the residue was subjected to chloroform/aq $NaHCO_3$ work-up. The yield of the crude product dried on Na_2SO_4 was nearly quantitative. It was used for 4-methoxytritylation of the amino group without further purification. ^{1}H NMR ($CDCl_3$): δ 8.14 (d, J = 7.5 Hz, 1H, H6), 6.00 (d, J = 1.1 Hz; 1H, H1′), 6.82 (d, J = 7.5 Hz, 1H, H5), 4.22 (dd, J = 8.0 and 5.1 Hz, 1H, H3′), 4.09 (dd, J = 11.8 and 1.8 Hz, 1H, H5′), 3.97 (m, 1H, H4′), 3.87 (dd, J = 11.8 and 1.6, 1H, H5″), 3.73 (dd, J = 5.1 and 1.1 Hz, 1H, H2′), 3.67 (s, 3H, 2′-OMe), 0.94 (s, 9H, Me_3C-Si), 0.13 (s, 3H, Me-Si), 0.13 (s, 3H, Me-Si). ESI-HRMS: $[M + H]^+$ obsd. 372.1908, calcd. 372.1956.

5′-*O*-(*tert*-Butyldimethylsilyl)-*N*^{4}-(4-methoxytrityl)-2′-*O*-methylcytidine (10). Compd. **9** (18.4 mmol; 6.84 g) was co-evaporated twice from dry pyridine and dissolved in the same solvent (20 mL). 4-Methoxytrityl chloride (18.4 mmol; 5.69 g) was added and the mixture was agitated at 45 °C for 24 h. MeOH (20 mL) was added, the mixture was evaporated to dryness and the residue was subjected to chloroform/aq $NaHCO_3$ work-up. Silica gel chromatography with DCM containing 2-5% MeOH gave compound **10** as a solid foam in 46% overall yield starting from 2′-*O*-methylcytidine. ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 7.7 Hz, 1H, H6), 7.26-7.33 (m, 6H, MMTr), 7.21-7.23 (m, 4H, MMTr), 7.13-7.15 (m, 2H, MMTr), 6.82-6.85 (m, 2H, MMTr), 6.77 (br. s, 1H, NH), 5.99 (s, 1H, H1′), 5.00 (d, J = 7.7 Hz, 1H, H5), 4.12 (m, 1H, H3′), 4.02 (dd, J = 11.9 and 1.2 Hz, 1H, H5′), 3.86-3.88 (m, 1H, H4′), 3.81 (dd, J = 11.9 and 1.2 Hz, 1H, H5″), 3.81 (s, 3H, MeO-MMTr), 3.72-3.74 (m, 4H, H2′ and 2′-OMe), 2.63 (br s, 1H, 3′-OH), 0.75 (s, 9H, Me_3C-Si), –0.03 (s, 3H, Me-Si), –0.05 (s, 3H, Me-Si). ^{13}C NMR ($CDCl_3$) δ 165.6 (C4), 158.7 (MMTr), 155.1 (C2), 144.4 (MMTr), 144.3 (MMTr), 140.9 (C6), 136.0 (MMTr), 130.0 (MMTr), 128.6 (MMTr), 128.3 (MMTr), 127.5 (MMTr), 113.6 (MMTr), 94.2 (C5), 87.6 (C1′), 83.9 (C2′), 83,7 (C4′), 70.5 (MMTr), 66.8 (C3′), 60.5 (C5′), 58.8 (2′-OMe), 55.2 (MMTr), 25.8 (TBDMS), 18.3 (TBDMS), –5.6 (TBDMS), –5.7 (TBDMS). ESI-HRMS: $[M + Na]^+$ obsd. 666.2189, calcd. 666.2178.

5′-*O*-(*tert*-Butyldimethylsilyl)-3′-*O*-levulinoyl-*N*^{4}-(4-methoxytrityl)-2′-*O*-methylcytidine (11). Levulinic acid (21.6 mmol; 2.51 g) was dissolved in dry dioxane and dicyclohexylcarbodiimide (11.1 mmol; 2.28 g) was added portionwise during 1 h at 0 °C. The mixture was allowed to warm up to reduce its viscosity and it was then filtrated to a solution of compd. 10 (8.46 mmol; 5.45 g) in pyridine (18 mL). The mixture was agitated overnight, evaporated to dryness and the residue was subjected to DCM/$NaHCO_3$ work-up. The organic phase was dried on Na_2SO_4, evaporated to dryness and the residue was purified by silica gel chromatography using DCM containing 1% MeOH as an eluent. Yield 86%. ^{1}H NMR ($CDCl_3$) δ 7.81 (d, J = 7.7 Hz, 1H, H6), 7.27-7.34 (m, 6H, MMTr), 7.22-7.23 (m, 4, MMTr), 7.14-7.15 (m, 2H, MMTr), 6.84-6.86 (m, 2H, MMTr), 6.80 (br. s, 1H, NH), 6.07 (d, J = 1.5 Hz, 1H, H1′), 4.99 (d, J = 7.7 Hz, 1H, H5), 4.97 (dd, J = 7.9 and 5.0 Hz, 1H, H3′), 4.21 (m, 1H, H2′), 3.99-4.01 (m, 2H, H4′ and H5′), 3.81 (s, 3H, MeO-MMTr), 3.70 (dd, J = 12.0 and 1.3 Hz, 1H, H5″), 3.57 (s, 3H, 2′-OMe), 2.63-2.83 (m, 4H, Lev), 2.21 (s, 3H, Lev), 0.74 (s, 9H, Me_3C-Si), –0.05 (s, 3H, Me-Si), –0.07 (s, 3H, Me-Si). ^{13}C NMR ($CDCl_3$) δ 206.1 (Lev), 172.0 (Lev), 165.5 (C4), 158.7 (MMTr), 155.1 (C2), 144.4 (MMTr), 144,3 (MMTr), 140.7 (C6), 136.0 (MMTr), 130.0 (MMTr), 128.6 (MMTr), 128.3 (MMTr), 127.5 (MMTr), 113.6 (MMTr), 94.4 (C5), 88.4 (C1′), 82.5 (C2′), 81,3 (C4′), 70.6 (MMTr), 69.1 (C3′), 60.8 (C5′), 58.9 (2′-OMe), 55.2 (MMTr), 37.8 (Lev), 29.8 (Lev), 27.8 (Lev), 25.7 (TBDMS), 18.2 (TBDMS), –5.7 (TBDMS), –5.8 (TBDMS).

3′-*O*-Levulinoyl-*N*^{4}-(4-methoxytrityl)-2′-*O*-methylcytidine (12). Compd. **11** (3.40 mmol; 2.52 g) was dissolved into a mixture THF (48 mL) and AcOH (9 mL) containing tetrabutylammonium fluoride (6.85 mmol; 1.79 g). The mixture was agitated for 2 days and then evaporated to dryness. The residue was dissolved into EtOAc (50 mL), washed with water, aq $NaHCO_3$ and brine, and dried on Na_2SO_4. Compd. **12** was obtained as a white foam in virtually quantitative yield. ^{1}H NMR ($CDCl_3$) δ 7.22-7.34 (m, 11H, H6 and MMTr), 7.12-7.15 (m, 2H, MMTr), 6.89 (br. s, 1H, NH), 6.83-6.85 (m, 2H, MMTr), 5.41 (d, J = 5.0 Hz, 1H, H1′), 5.31 (dd, J = 4.8 and 4.7, 1H, H4′), 5.07 (d, J = 7.6 Hz, 1H, H5), 4.58 (dd, J = 4.8 and 5.0 Hz, 1H, H3′), 4.18 (m, 1H, H2′), 3.90 (d, J = 12.7 Hz, 1H, H5′), 3.81 (s, 3H, MeO-MMTr), 3.71 (dd, J = 12.7 and 4.7 Hz, 1H, H5″), 3.45 (s, 3H, 2′-OMe), 2.75-2.80 (m, 2H, Lev), 2.63-2.66 (m, 2H, lev), 2.20 (s, 3H, Lev). ESI-HRMS: $[M + H]^+$ obsd. 628.2603, calcd. 628.2661; $[M + Na]^+$ obsd. 650.2434, calcd. 650.2481.

3′-*O*-Levulinoyl-*N*^{4}-(4-methoxytrityl)-2′-*O*-methylcytidine 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (13). Compd. **12** (2.58 mmol; 1.62 g) dried on P_2O_5 for 2 days was dissolved in dry pyridine (5 mL) and diphenylphosphite (3.09 mmol; 595 μL) was added under nitrogen. After half an hour, carefully dried L-alanine methyl ester (3.94 mmol; 0.55 g) in a mixture of dry pyridine (1 mL) and MeCN (6 mL) was added. CCl_4 (15 mL) and triethylamine (18.1 mmol; 2.54 mL) was added

and the reaction was allowed to proceed for 70 min. Volatiles were removed under reduced pressure and the residue was purified by silica gel chromatography increasing the MeOH content of DCM from 1 to 10% in a stepwise manner. Compd. **13** was obtained as white foam in 70% yield. ^{1}H NMR ($CDCl_3$) mixture of R_P and S_P diastereomers δ 7.02-7.35 (m, 17H, MMTr and Ph), 6.80-6.85 (m, 3H, MMTr and N^4H), 5.99 and 6.02 (2×d, J = 3.2 Hz, 1H, H1′), 4.90-5.00 (m, 2H, H3′ and H4′), 3.88-4.43 (m, 4H, H5, H2′, H5′, H5″), 3.80 (s, 3H, MMTr), 3.68-3.75 (m, 1H, H$^\alpha$-Ala), 3.63 and 3.64 (2×s, 3H, MeO-Ala), 3.46 and 3.52 (2×s, 3H, 2′-OMe), 2.74-2.81 (m, 2H, Lev), 2.59-2.64 (m, 2H, Lev), 2.19 and 2.20 (2×s, 3H, Lev), 1.88 (br s, 1H, NH-P), 1.27 and 1.31 (2×d, J = 7.1 Hz, Me Ala). ESI-HRMS: [M + H]$^+$ obsd. 869.3111, calcd. 869.3165; [M + Na]$^+$ obsd. 891.2940, calcd. 891.2985.

2′-*O*-Methylcytidine 5′-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (4). Compd. **13** (1.81 mmol; 1.57 g) was dissolved in a mixture of hydrazine hydrate (7.2 mmol; 350 µL), pyridine (11.5 mL) and AcOH (2.88 mL) and the reaction was allowed to proceed for 5 h. Volatiles were removed under reduced pressure and the residue was dissolved in DCM (50 mL) and washed with water, aq $NaHCO_3$ and brine. The organic phase was dried on Na_2SO_4, evaporated to dryness and the residue was purified by silica gel chromatography using DCM containing 4-6% MeOH as an eluent. The purified product was dissolved 80% aq AcOH (8 mL) and the mixture was allowed to proceed at 55 °C for 2 h and additionally at 65 °C for 4.5 h. The mixture was evaporated to dryness and the residue was coevaporated twice from water and then purified by silica gel chromatography using gradient elution from 7 to 20% MeOH in DCM. The overall yield from **13** was 50%. ^{1}H NMR ($CDCl_3$) mixture of two diastereomers δ 7.64 and 7.68 (2×d, J = 7.4, 1H, H6), 7.26-7.33 (m, 2H, Ph), 7.20-7.24 (m, 2H, Ph), 7.13-7.16 (m, 1H, Ph), 6.32 (br s, 2H, NH_2), 5.90 and 5.94 (2×s, 1 H, H1′), 5.69 and 5.82 (2×d, J = 7.4, 1H, H5), 4.35-4.55 (m, 2H, H5′ and H5″), 4.12-4.18 (m, 2H, H3′ and H4′), 3.98-4.08 (m, 2H, α-H-Ala and 3′-OH), 3.72-3.76 (m, 1H, 2′-OMe), 3.67 and 3.68 (2×s, 3H, MeO-Ala), 3.58 and 3.60 (2×s, 3H, 2′-OMe), 2.45 (br s, 1H, NH-P), 1.37 and 1.39 (2×d, J = 7.2 Hz, 3H, Me-Ala). ^{13}C NMR ($CDCl_3$) δ174.2 (C=O Ala), 166.0 (C4), 155.9 (C2), 150.5 (Ph), 140.6 (C6), 129.8 (Ph), 125.1 (Ph), 120 (Ph), 95.1 (C5), 88.4 (C1′), 83.4 (C2′), 81.4 (C4′), 68.1 (C3′), 65.1 (C5′), 58.6 (2′-OMe), 52.5 (MeO-Ala), 50.3 (C$^\alpha$-Ala), 20.7 (Me-Ala). ^{31}P NMR δ 3.1 and 3.3. ESI-HRMS [M + H]$^+$ obsd. 499.1590, calcd. 499.1583; [M + Na]$^+$ obsd. 521.1438, calcd. 521.1408, [M + K]$^+$ obsd. 537.1149, 537.1147.

2′,3′-Di-*O*-levulinoylribavirin 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (14a). Diphenylphosphite was dissolved into dry pyridine (1.0 mL) and diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate[25] (0.29 mmol; 56 µL) in dry pyridine (1 mL) was added portion wise. After 30 min, compd. **7** (0.34 mmol; 0.151 g) in dry pyridine (1.5 mL) was added drop wise and the reaction was allowed to proceed for 2 h. L-Alanine methyl ester (0.29 mmol; 41 mg) in dry pyridine (250 µL) was added, followed by dry MeCN (3.5 mL), CCl_4 (1.8 mL) and triethyl amine (1.45 mmol; 205 µL). The reaction was allowed to proceed for 45 min and the volatiles were then removed under reduced pressure. Crude compd. **14** was purified by silica gel chromatography using gradient elution from 3 to 15% MeOH in DCM. Yield 44%. ^{1}H NMR ($CDCl_3$) mixture of R_P and S_P diastereomers δ 8.41 and 8.46 (2×s, 1H, H5), 740 and 7.46 (2×br s, 1H, NH_2), 6.06 and 6.08 (2×d, J = 4.5 Hz, 1H, H1′), 5.89 (br s, 1H, NH_2), 5.71 and 5.73 (2×dd, J = 4.5 and 4.7 Hz, 1H, H2′), 5.56 and 5.58 (2×dd, J = 4.7 and 4.9 Hz, 1H, H3′), 4.43-4.58 (m, 5H, H4′,CH_2OAc and CH_2OP), 4.18-4.36 (m, 6H, H5′, H5″, CH_2CH$_3$), 3.90-3.96 (m, H$^\alpha$-Ala), 3.71 and 3.73 (2×s, 3H, MeO-Ala), 2.76-2.83 (m, 4H, Lev), 2.57-2.68 (m, 4H, Lev), 2.18 and 2.19 (2×s, 3H, Lev), 2.20 and 2.21 (2×s, 3H, Lev), 2.05 and 2.06 (2×s, 3H, Ac), 1.84 (br s, 1H, NH-P), 1.35 (d, J = 7.0 Hz, 3H, Me Ala), 1.24-1.27 (m, 6H, CH_2CH_3). ^{31}P NMR ($CDCl_3$) = 7.2 and 7.4. ESI-HRMS: [M + H]$^+$ obsd. 850.2712, calcd. 850.2761; [M + Na]$^+$ obsd. 872.2521, calcd. 872.2581.

2′,3′-Di-*O*-levulinoylribavirin 5′-{*O*-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (14b). Diphenylphosphite was dissolved into dry pyridine (2.0 mL) and diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate[25] (1.95 mmol; 0.57 g) in dry pyridine (2 mL) was added portion wise. After 30 min, compd. **7** (2.34 mmol; 1.03 g) in dry pyridine (2 mL) was added drop wise and the reaction was allowed to proceed for 2 h. L-Alanine methyl ester (3.65 mmol; 0.51 g) in dry pyridine (1 mL) was added, followed by dry MeCN (7 mL), CCl_4 (11 mL) and triethyl amine (13.7 mmol; 1.9 mL). The reaction was allowed to proceed for 75 min and the volatiles were then removed under reduced pressure. Crude compd. **14b** was purified by silica gel chromatography using gradient elution from 3 to 8% MeOH in DCM. Yield 37%. ^{1}H NMR ($CDCl_3$) mixture of R_P and S_P diastereomers δ 8.41 and 8.47 (2×s, 1H, H5), 7.44 (br s, 2H, NH_2), 6.12 (br s, 1H, NH_2), 6.08 (m, 1H, H1′), 5.71 (m, 1H, H2′), 5.56, (m, 1H, H3′), 5.24 (2×s, 2H, OCH_2OAc), 4.45-4.52 (m, 3H, H4′,CH_2OAc and), 4.16-4.26 (m, 6H, H5′, H5″, 2×CH_2CH$_3$), 4.10-4.13 (m, 2H, CH_2OP), 3.88-3.99 (m, H$^\alpha$-Ala), 3.66 and 3.71 (2×s, 3H, MeO-Ala), 2.71-2.83 (m, 4H, Lev), 2.57-2.68 (m, 4H, Lev), 2.17 and 2.18 (2×s, 3H, Lev), 2.19 and 2.20 (2×s, 3H, Lev), 2.08 (s, 3H, Ac), 1.87 (2×s, 1H, NH-P), 1.32-1.37 (m, 3H, Me Ala), 1.22-1.25 (m, 6H, CH_2CH_3). ESI-HRMS: [M + H]$^+$ obsd. 850.2712, calcd. 850.2761; [M + Na]$^+$ obsd. 872.2521, calcd. 872.2581.

Ribavirin 5′-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (1a). Compd. **14a** (0.36 mmol; 0.31 g) was added into a mixture of hydrazine hydrate (3.98 mmol; 124 µL), pyridine (4.0 mL) and AcOH (1.0 mL). After 30 min, acetone was added to quench the unreacted hydrazine and the mixture was evaporated to dryness. The crude product was purified by silica gel chromatography increasing the MeOH content of DCM in a stepwise manner from 5% to 8% and then to 20%. The product obtained was still subjected to RP-HPLC purification (Hypersil ODS2, 21.2 × 250 mm, 5 µm) using a gradient elution from 25% aq MeCN to 40% aq MeCN. Yield 35%.^{1}H NMR (CD_3OD + D_2O) mixture of R_P and S_P diastereomers δ 8.72 and 8.74 (2×s, 1H, H5), 6.01 and 6.02 (2×d, J = 3.0 Hz, 1H, H1′), 4.41-4.59 (m, 6H, H2′, H3′, CH_2OAc and CH_2OP), 4.14-4.31 (m, 7H, H4′, H5′, H5″, CH_2CH$_3$), 3.78 and 3.84 (2×dd, J = 9.3 and 7.2 Hz, H$^\alpha$-Ala), 3.72 and 3.74 (2×s, 3H, MeO-Ala), 2.07 and 2.08 (2×s, 3H, Ac), 1.32 and 1.34 (d, J = 7.2 Hz, 3H, Me Ala), 1.24-1.28 (m, 6H, CH_2CH_3). ^{13}C NMR (CD_3OD + D_2O) δ 174.7 (C=O Ala),

171.6 (*C*OOEt), 167.0 (O*C*OMe), 161.9 (*C*ONH$_2$), 157.0 (C3), 145.7 (C5), 92.1 (C1′), 83.0 (C4′), 74.6 (C2′), 70.2 (C3′), 66.4 (C5′), 61.3 (*C*H$_2$CH$_3$), 58.0 (spiro C), 52.0 (MeO-Ala), 50.0 (C$^{\alpha}$-Ala), 19.5 (Ac), 19.0 (Me Ala), 13.0 (CH$_3$*C*H$_3$). ^{31}P NMR (CD$_3$OD + D$_2$O) = 8.1 and 8.0. ESI-HRMS: [M + H]$^+$ obsd. 654.2022, calcd. 654.2018; [M + Na]$^+$ obsd. 676.1876, calcd. 676.1838; [M + K]$^+$ obsd. 692.1588, calcd. 692.1577.

Ribavirin 5′-{*O*-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl]-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]-phosphoramidate} (1b). Compd. **14b** (0.73 mmol; 0.63 g) was dissolved into dichloromethane (3.0 mL). Hydrazine acetate (2.4 mmol; 0.22 g) dissolved in methanol (1 mL) was added. After four hours, acetone was added to quench the unreacted hydrazine and the mixture was evaporated to dryness. The crude product was purified by silica gel chromatography increasing the MeOH content of DCM in a stepwise manner from 8% to 10%. The silica gel purification was repeated using gradient elution from 5 to 20% MeOH in DCM. Yield 44%. ^{1}H NMR (CD$_3$Cl$_3$) mixture of R_P and S_P diastereomers δ 8.57 and 8.53 (2×s, 1H, H5), 7.65 and 7.60 (d, 1H, N*H*), 6.97 and 6.91 (d, 1H, N*H*), 5.99 (s, 1H, H1′), 5.31 (m, 1H, H3′), 5.22-5.25 (2×s, 2H, OC*H*$_2$OAc), 4.57 (m, 1H, H2′), 4.08-4.50 (m, 10H, C*H*$_2$OCH$_2$, C*H*$_2$OP, H5′, H5″, 2×C*H*$_2$CH$_3$), 3.87 and 3.80 (2×dd, J = 7.5 Hz, H$^{\alpha}$-Ala), 3.71 and 3.66 (2×s, 3H, MeO-Ala), 2.08 and 2.07 (2×s, 3H, Ac), 1.36 and 1.31 (2×d, J = 7.1 Hz, 3H, Me Ala), 1.21-1.26 (m, 6H, CH$_2$C*H*$_3$). ^{13}C NMR (CD$_3$Cl$_3$) δ 170.6 (C=O Ala), 169.1 (O*C*OMe), 166.9 (*C*OOEt), 161.5 (*C*ONH$_2$), 157.0 (C3), 145.1 (C5), 92.5 (C1′), 89.0 (O*C*H$_2$OAc), 83.1 (C4′), 75.2 (C2′), 70.6 (C3′), 66.4 (C5′), 64.0 (*C*H$_2$O), 62.1 (*C*H$_2$CH$_3$), 58.9 (spiro C), 52.5 (MeO-Ala), 50.0 (C$^{\alpha}$-Ala), 20.6 (Ac), 20.5 (Me Ala), 13.9 (CH$_3$*C*H$_3$). ^{31}P NMR (CD$_3$OD + D$_2$O) = 8.1 and 8.0. ESI-HRMS: [M + H]$^+$ obsd. 654.2022, calcd. 654.2018; [M + Na]$^+$ obsd. 676.1876, calcd. 676.1838; [M + K]$^+$ obsd. 692.1588, calcd. 692.1577.

3′-*O*-Levulinoyl-*N*^{4}-(4-methoxytrityl)-2′-*O*-methylcytidine 5′-[*O*-3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-(*S*-2-methoxy-1-methyl-2-oxoethyl)]phosphoramidate (15). Diphenylphosphite (2.83 mmol; 545 μL) was dissolved in dry pyridine (2.0 mL) and diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (2,36 mmol; 0.62 g) in dry pyridine (2.0 mL) was dropwise added under nitrogen. After 40 min from the beginning of the reaction, compd. **12** (3.30 mmol; 2.07 g) in dry pyridine (4 mL) was added dropwise under nitrogen. After 2.5 h, methyl ester of L-alanine (2.85 mmol; 0.398 g) in dry pyridine (1 mL) was added. Finally, dry MeCN (9.0 mL), CCl$_4$ (14.0 mL) and distilled triethylamine (16.5 mmol; 2.33 mL) were added to the mixture and the reaction was allowed to proceed for 1 h. Volatiles were removed under reduced pressure and the residue was dissolved in DCM (50 mL) and washed with water, aq NaHCO$_3$ and brine. The organic phase was dried on Na$_2$SO$_4$ and concentrated to a yellow oil. Purification by silica gel chromatography using DCM containing 2-3% MeOH as an eluent, gave compd. **15** in 24% yield. ^{1}H NMR (CDCl$_3$), mixture of S_P and R_P diastereomers δ 7.21-7.33 (m, 11H, H6 and MMTr), 7.13-7.15 (m, 2H, MMTr), 6.89 (br. s, 1H, NH), 6.82-6.84 (m, 2H, MMTr), 5.96 and 5.97 (2×d, J = 2.5 Hz, 1H, H1′), 5.41 and 5.30 (2×d, J = 5.0 Hz, 1H, H4′), 5.13 and 5.14 (2×d, J = 5.0, 1H, H3′), 5.06 (d, J = 7.6 Hz, 1H, H5), 4.84-4.93 (m, 1H, H2′), 4.57-4.60 (m, 2H, C*H*$_2$OAc), 4.48-4.52 (m, 2H, C*H*$_2$OP), 4.37-4.48 (m, 2H, H5′ and H5″), 4.10-4.25 (m, 6H, OC*H*$_2$Me), 3.98 (m, 1H, α-H-Ala), 3.80 (s, 3H, MeO-MMTr), 3.63 and 3.64 (2×s, 3H, MeO-Ala), 3.49 (s, 3H, 2′-OMe), 2.73-2.78 (m, 2H, Lev), 2.61-2.65 (m, 2H, Lev), 2.19 (s, 3H, Lev), 2.00 and 2.01 (2×s, 3H, OAc), 1.77 (br s, NH-Ala), 1.24-1.26 (m, 9H, Me-Ala and-CH$_2$C*H*$_3$).

2′-*O*-Methylcytidine 5′-[*O*-3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-*N*-(*S*-2-methoxy-1-methyl-2-oxoethyl)]phosphoramidate (2). Compd. **15** (0.73 mmol; 0.760 g) was dissolved into a mixture of hydrazine hydrate (1.59 mmol; 50 μL), pyridine (1.6 mL) and AcOH (0.4 mL). The reaction was allowed to proceed for 75 min. Unreacted hydrazinium acetate was then quenched with acetone and volatiles were removed under reduced pressure and the residue was purified by silica gel chromatography using a 1 : 10 mixture (v/v) of MeOH and EtOAc as eluent. The residue (410 mg) obtained by evaporation to dryness was dissolved in 80% aq AcOH and the reaction was allowed to proceed overnight. The product was purified by RP-HPLC on a SunFire prep C18 column (10 × 250 mm, 5 μm) using a stepwise gradient elution from 20% MeCN in water to 40% MeCN in H$_2$O. The yield was 10%. ^{1}H NMR (CDCl$_3$), mixture of S_P and R_P diastereomers δ 7.71 and 7.74 (2×d, J = 7.5 Hz, 1H, H6), 5.90-5.94 (m, 2H, H1′ and H5), 4.57-4.66 (m, 2H, C*H*$_2$OAc), 4.41-4.52 (m, 2H, C*H*$_2$OP), 4.35-4.39 (m, 1H, H5′), 4.21-4.28 (m, 5H, H5″ and OC*H*$_2$Me), 4.13-4.16 (m, 1H, H3′), 4.09-4.10 (m, 1H, H4′), 3.92-3.94 (m, 1H, α-H-Ala), 3.80-3.82 (m, 1H, H2′), 3.73 and 3.75 (2×s, 3H, MeO-Ala), 3.67 (s, 3H, 2′-OMe), 2.06 and 2.07 (2×s, 3H, OAc), 1.41 and 1.42 (d, J = 7.1 Hz, 3H, Me-Ala), 1.24-1.29 (m, 6H,-CH$_2$C*H*$_3$). ^{13}C NMR (CDCl$_3$) δ 174.3 (C=O Ala), 174.2 (C=O Ac), 170.5 (COOEt), 166.1 (C4), 166.0 (C2), 140.6 (C6), 94.7 (C5), 88.5 (C1′), 83.4 (C2′), 81.4 (C4′), 68.0 (C3′), 64.2 (C5′), 64.0 (*C*H$_2$OP), 62.3 (*C*H$_2$Me), 61.5 (*C*H$_2$OAc), 58.6 (MeO-2′), 58.1 (spiro C), 52.5 (MeO-Ala), 50.0 (C$^{\alpha}$-Ala), 20.8 (Ac), 20.7 (Me-Ala), 14.0 (CH$_2$*C*H$_3$). ^{31}P NMR (CDCl$_3$) δ 7.5 and 7.7. ESI-HRMS: [M + H]$^+$ obsd. 667.2212, calcd. 667.2222; [M + Na]$^+$ obsd. 689.2023, calcd. 689.2042.

Kinetic measurements

Reactions were carried out in sealed tubes immersed in a thermostated water bath (37.0 ± 0.1 °C). The hydroxonium ion concentration of the reaction solutions (3 mL) was adjusted with sodium hydroxide and *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES) and glycine buffers. The ionic strength of the solutions was adjusted to 0.1 mol L^{-1} with sodium chloride. The hydronium ion concentrations of the buffer solutions were calculated with the aid of the known pK_a values of the buffer acids under the experimental conditions. Low buffer concentration was used (60 mmol L^{-1}). The initial substrate concentration was *ca.* 0.1 mmol L^{-1}. The acetyl group of **2** was removed with Porcine Liver Esterase (7.5 mg, 16 units/mg) in an acetic acid buffer (0.2/0.04 mol L^{-1}). After 2 days treatment at 37 °C, the pH of the solution was adjusted to **2** by adding 360 μL of 1 mol L^{-1} aqueous hydrogen chloride and the solution was filtered with SPARTAN 13A filters (0.2 μm). To start the kinetic run with **16**, the pH of the solution was adjusted to 7.5 with HEPES. The aliquots withdrawn (200 μL) were made acidic (pH 2) with aqueous hydrogen chloride and cooled in an ice-bath. Their composition was analyzed on an ODS Hypersil C18 column (4 × 250 mm, 5 μm), using a mixture of acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) and

MeCN, containing ammonium chloride (0.1 mol L^{-1}). A good separation of the product mixtures of **2** and **4** was obtained on using a 5 min isocratic elution with the buffer containing 2% MeCN, followed by a linear gradient (23 min) up to 40% MeCN. The observed retention times (t_R/min) for the starting materials were 30.6 and 30.8 (**2**, two diastereomers) and 28.0 and 28.5 (**4**, two diastereomers) and those for the hydrolysis products were 28.5 and 28.8 (**16**, 2 diastereomers), 27.8 and 28.0 (**18**, two diastereomers), 23.0 (21), 16.8 (17), 11.2 (20) at a flow rate 1 mL min^{-1}. Signals were recorded on a UV-detector at a wavelength of 267 nm. The reaction products were identified by the mass spectra (LC/MS). A mixture of water and MeCN containing formic acid (0.1%) was used as an eluent.

Calculation of rate constants

First-order rate constants, k_d, for the non-enzymatic hydrolysis of compounds **2** and **4** were calculated by applying the first-order rate-law to diminution of the concentration of the starting material. The first-order rate constant for the conversion of **16**, obtained by enzymatic deacetylation, to **17** (k_2 in Scheme 7) to was determined similarly. The rate constant for the conversion of compound **19** to **20** (k_6) could not be determined, but it is in all likelihood approximately equal to k_2.

On using **2** as a starting material, **17** and **18** accumulated as intermediates at pH 9.0 and 10.0. Application of the rate-law of two consecutive first-order reactions on the concentration of these species allowed determination of rate constants k_1 and k_4 (Scheme 7) by least-squares fitting of the data to eqn (1) and (2), respectively. The meaning of rate constant k_1, k_3, k_4 and k_5 is indicated in Scheme 7. Rate constant k_d is the sum of k_1 and k_4.

$$[\mathbf{17}] = k_1 \frac{e^{-k_d t} - e^{-k_3 t}}{k_3 - k_d} [\mathbf{2}]_0 \quad (1)$$

$$[\mathbf{18}] = k_4 \frac{e^{-k_d t} - e^{-k_5 t}}{k_5 - k_d} [\mathbf{2}]_0 \quad (2)$$

Preparation of the cell extract

3×10^7 human prostate carcinoma cells (PC3) were treated with 10 ml of RIPA-buffer [15 mM Tris-HCl pH 7.5, 120 nM NaCl, 25 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.1% deoxycholic acid, 0.5% Triton X-100, 0.5% PMSF supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics GmBH, Germany)] at 0 °C for 10 min. Most of the cells were disrupted mechanically. The cell extract obtained was centrifuged (900 rpm, 10 min) and the pellet was discarded. The extract was stored at –20 °C.

Stability of protected nucleoside phosphoramidates (1a, 2, 3) in the PC3 cell extract

Compound **1a**, **2** or **3** (0.3 mg) was added into a 1 : 2 mixture of the PC3 cell extract and HEPES buffer (0.02 mol L^{-1}, pH 7.5, I = 0.1 mol L^{-1} with NaCl) and the mixture was kept at 35 ± 1 °C. Aliquots of 200 μL were withdrawn at appropriate intervals into tubes containing aqueous hydrogen chloride (10 μL 1 mol L^{-1}), cooled on an ice bath and passed through SPARTAN 13A filters (0.2 μm). The samples were analyzed by RP HPLC on a Phenomenex Gemini C18 column (4.6 × 150 mm, 5 μm, 110 Å, flow rate 1 mL min^{-1}) using a mixture of ammonium acetate buffer (50 mmol L^{-1}) and MeCN as an eluent. A good separation of the product mixtures was obtained on using isocratic elution with a buffer containing 15% of MeCN in the case of **3** and 20% in the case of **1a** and **12**. The observed retention times (t_R/min) for the diastereomers of the starting material were 11.78 and 15.97 (**3**), 20.09 and 24.34 (**1a**) and 31.22 and 35.39 (**2**). The retention time for the main hydrolysis product of **1a** was 2.35 (**23**). Signals were recorded on a UV-detector at a wavelength of 220 nm in the case of **1a** and **3** and at 270 nm in the case of **2**. The isolated reaction products were identified by mass spectroscopy (ESI-TOF). The decomposition of **1a-3**, was additionally followed by HPLC-MS/MS on a Phenomenex Gemini C18 column (2.0 × 150 mm 5 μm 110 Å, flow rate 0.2 mL min^{-1}), applying the following gradient elution. Buffer A: a 1 : 1 mixture of 0.1% formic acid and MeCN, buffer B: 0.1% formic acid; 10 min isocratic elution with 4% A in B, followed by a linear gradient to 100% A in 40 min. The retention times (t_R/min) observed for the starting materials were 34.3 and 33.7 (**2**, two diastereomers). The retention times for the hydrolysis products of **2** were 32.9 and 32.5 (**18**, two diastereomers), 23.2 (**17**), and 16.6 (**20**). The flow rate was 0.2 mL min^{-1} and the signals were recorded on a UV-detector at a wavelength of 230 nm (**1a** and **3**) and 270 nm (**2**).

Stability of protected nucleoside phosphoramidates (1a, 1b, 3) towards porcine liver esterase

0.02 mg (3 units) of BioChemika Hog Liver Esterase (46058) was dissolved in 3 mL of HEPES buffer (0.02 mol L^{-1}, pH 7.5, I = 0.1 mol L^{-1} with NaCl). Compound **1a**, **b** or **3** was added and the mixture was kept at 35 ± 1 °C. The aliquots were withdrawn and analyzed as described above for the cell extract. A good separation of the product mixtures was obtained on using isocratic elution with a buffer containing 15% MeCN in the case of **3** and 20% in the case of **1a** and **1b**. The observed retention times (t_R/min) for the diastereomers of the starting material were 11.78 and 15.97 (**3**), 20.09 and 24.34 (**1a**) and 26.69 and 32.64 (**1b**). The retention time (t_R/min) for the main hydrolysis product of **1a** was 2.35 (**23**). With **1b**, the retention times (t_R/min) for the diastereomers of **22** were 6.77 and 7.87. Signals were recorded on a UV-detector at a wavelength of 220 nm. The decomposition of **1a**, **1b** and **3** and was additionally followed by HPLC-MS/MS on a Phenomenex Gemini C18 column (2.0 × 150 mm, 5 μm, 110 Å, flow rate 0.2 mL min^{-1}), applying the following gradient elution. Buffer A: a 1 : 1 mixture of 0.1% formic acid and MeCN, buffer B: 0.1% formic acid; 10 min isocratic elution with 4% A in B, followed by a linear gradient to 40% A in B in 40 min (with **3**), to 60% A in B in 40 min (with **1a**) or to 100% A in B in 50 min (with **1b**). The retention times (t_R/min) observed for the starting materials were 48.5 and 51.3 (**3**, two diastereomers), 50.4 and 51.4 (**1a**, two diastereomers) and 39.1 and 39.9 (**1b**, two diastereomers). The retention time (t_R/min) for the main hydrolysis product of **1b** was 23.24 (**23**). The flow rate was 0.2 mL min^{-1} and the signals were recorded on a UV-detector at a wavelength of 230 nm.

Conclusions

It has been shown that 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups

are potential biodegradable protecting groups of nucleoside 5′-phosphoramidates derived from L-alanine methyl ester. Treatment with either porcine carboxyesterase or whole cell extract of human prostate carcinoma cells (PC3) results in hydrolytic removal of the acetyl group and this triggers a series of non-enzymatic reactions that remove the remnants of the protecting group releasing the phosphoramidate monoanion. With the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, hydrolysis of the alanine methyl ester linkage competes with the removal of the protecting group, representing about 20% of the total disappearance of the pro-drug. The 3-acetyloxymethoxy group is removed one order of magnitude faster and the alanine methyl ester linkage, hence, remains intact. The removal of this group also occurs more readily than the conversion of *O*-phenyl protected phosphoramidates derived from L-alanine methyl ester to unprotected alanine-derived phosphoramidates. The difference in reaction rates is at a low enzyme concentration (1 U mL^{-1}) approximately 10-fold. Although these studies are preliminary in nature and insufficient to prove the applicability of 3-acetyloxymethoxy-2,2.bis(ethoxycarbonyl)propyl group as a biodegradable protecting group for nucleoside phosphoramidates, the results appear encouraging enough to urge comparative studies with previously described pro-drugs on cell lines.

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